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<b>(21) International Application Number:</b> PCT/US96/15990 <b>(22) International Filing Date:</b> 3 October 1996 (03.10.96) <b>(30) Priority Data:</b> 08/539,142 4 October 1995 (04.10.95) US <b>(71) Applicant:</b> IMMUNEX CORPORATION [US/US]; 51 University Street, Seattle, WA 98101 (US). <b>(72) Inventors:</b> BRASEL, Kenneth; 7343 Jones N.W., Seattle, WA 98117 (US). LYMAN, Stewart, D.; 312 North 45th Street, Seattle, WA 98103 (US). MARASKOVSKY, Eugene; 4123 Evanston Avenue North, Seattle, WA 98103 (US). McKENNA, Hilary, R.; Apartment 32, 3809 Whitman Avenue North, Seattle, WA 98103 (US). LYNCH, David, H.; 14180 N. Madison Avenue N.E., Bainbridge Island, WA 98110 (US). <b>(74) Agent:</b> MALASKA, Stephen, L.; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).		<b>(81) Designated States:</b> AL, AU, BB, BG, BR, CA, CN, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LS, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DENDRITIC CELL STIMULATORY FACTOR  <b>(57) Abstract</b>  Flt3-ligand can be used to generate large numbers of dendritic cells from hematopoietic progenitor and stem cells. Flt3-ligand can be used to augment immune responses in vivo, and expand dendritic cells ex vivo. Such dendritic cells can then be used to present tumor, viral or other antigens to naive T cells, can be useful as vaccine adjuvants.		

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**TITLE**  
**DENDRITIC CELL STIMULATORY FACTOR**

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**FIELD OF THE INVENTION**

The present invention relates to a dendritic cell stimulatory factor, to methods of enhancing an immune response in vivo, methods of expanding dendritic cells ex vivo, and to preparations of purified dendritic cells, and to dendritic cell populations useful in the manipulation of T cell-mediated and B-cell mediated immune responses.

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**BACKGROUND OF THE INVENTION**

The objective of vaccination is to provide effective immunity by establishing adequate levels of antibody and a primed population of cells that can rapidly expand on renewed contact with antigen. The first contact with antigen during vaccination must not be injurious to the recipient and thus usually consists of pathogenically-deficient antigen.

A frequent difficulty with active immunization protocols is that the vaccine antigen does not possess sufficient immunogenicity to promote a strong immune response, and therefore a sufficient level of protection against subsequent challenge by the same antigen. In addition, certain antigens may elicit only weak cell-mediated or antibody response. For many antigens, both a strong humoral response and a strong cell-mediated response is desirable.

For decades, researchers have experimented with diverse compounds to increase the immunogenicity of vaccines. Immunopotentiators, also known as adjuvants, of vaccines are compositions of matter that facilitate a strong immune response to a vaccine. In addition, the relatively weak immunogenicity of certain novel recombinant antigens has required adjuvants to be more potent. Vaccine adjuvants have different modes of action, affecting the immune response both quantitatively and qualitatively. Such modes of action can be by mobilizing T cells, acting as depots and altering lymphocyte circulation so that these cells remain localized in draining lymph nodes. They may also serve to focus antigen at the site of immunization, thereby allowing antigen specific T cells and B cells to interact more efficiently with antigen-presenting cells. They may also stimulate proliferation and differentiation of T cells and have effects on B cells, such as enhancing the production of different Ig isotypes. Further, adjuvants may stimulate and affect the behavior of antigen-presenting cells, particularly macrophages, rendering them more effective for presenting antigen to T cells and B cells.

Dendritic cells are a rare and heterogeneous cell population with distinctive morphology and a widespread tissue distribution. A discussion of the dendritic cell system and its role in immunogenicity is provided by Steinman, R.M., *Annu. Rev. Immunol.*, 9:271-296 (1991), incorporated herein by reference. Dendritic cells display an unusual cell surface and can be characterized by the presence of the cell surface markers CD1a<sup>+</sup>, CD4<sup>+</sup>, CD14<sup>-</sup> CD86<sup>+</sup>, CD11c<sup>+</sup>, DEC-205<sup>+</sup>, CD14<sup>+</sup> or HLA-DR<sup>+</sup>. Dendritic cells have a high capacity for sensitizing MHC-restricted T cells and provide an effective pathway for presenting antigens to T cells in situ, both self-antigens during T cell development and foreign antigens during immunity. Thus, there is growing interest in using dendritic cells ex vivo as tumor or infectious disease vaccine adjuvants. See, for example, Romani, et al., *J. Exp. Med.*, 180:83 (1994). The use of dendritic cells as immunostimulatory agents has been limited due to the low frequency of dendritic cells in peripheral blood, the limited accessibility to lymphoid organs and the dendritic cells' terminal state of differentiation. Dendritic cells originate from CD34<sup>+</sup> bone marrow progenitors, and the proliferation and maturation of dendritic cells can be enhanced by the cytokines GM-CSF (sargramostim, Leukine<sup>®</sup>, Immunex Corporation, Seattle, Washington), TNF- $\alpha$ , c-kit ligand (also known as stem cell factor (SCF), steel factor (SF), or mast cell growth factor (MGF)) and interleukin-4. Therefore, an agent that stimulated the generation of large numbers of functionally mature dendritic cells in vivo or in vitro would be of wide importance.

### SUMMARY OF THE INVENTION

Flt3-ligand ("flt3-ligand" or "flt3-L") is known to affect hematopoietic stem and progenitor cells. It was surprisingly found that flt3-ligand can also potently stimulate the generation of downstream or intermediate, cells such as myeloid precursor cells, monocytic cells, macrophages, B cells, and dendritic cells from CD34<sup>+</sup> bone marrow progenitors and stem cells. The present invention pertains to a method of mobilizing dendritic cells in vivo, expanding dendritic cells ex vivo and to purified preparations of dendritic cells. The preparation of purified dendritic cells according to the invention would potentially find use as vaccine adjuvants. Also included within the embodiments of the invention is a method of preparing antigen-specific T cells using the dendritic cells mobilized with flt3-ligand.

The invention provides for the use of an effective amount of flt3-ligand to increase or mobilize the numbers of intermediate cells in vivo, for example, in the patient's peripheral blood, tissues or organs. While the invention relates to the generation of large numbers of such downstream and intermediate cells (e.g., myeloid cells, monocytic cells and macrophages) from CD34<sup>+</sup> cells using flt3-ligand, the focus is particularly on dendritic cells. By increasing the quantity of the patient's dendritic cells, such cells may themselves be used to present antigen to T cells. For example, the antigen may be one that already

exists within the patient, such as a tumor antigen, or a bacterial or viral antigen. Flt3-ligand may be used, therefore, to increase the numbers of dendritic in vivo to boost a patient's immune response against existing antigens. Alternatively, flt3-ligand may be administered prior to, concurrently with or subsequent to administration of an antigen to a patient for immunization purposes. Thus, as a vaccine adjuvant, flt3-ligand can generate large quantities of dendritic cells and other intermediate cells in vivo to more effectively present the antigen. The overall response is a stronger and improved immune response and more effective immunization to the antigen.

The invention also provides a method of generating large quantities of dendritic cells ex vivo. Following collection of the patient's CD34<sup>+</sup> hematopoietic progenitors and stem cells, flt3-ligand can be used to expand such cells in vitro (also known as ex vivo expansion) and to drive such CD34<sup>+</sup> cells to differentiate into dendritic cells of the lymphoid or myeloid lineage. The resulting collection of dendritic cells can be administered to a patient to provide a stronger and improved immune response to an antigen. Alternatively, the resulting dendritic cells find use as a vaccine adjuvant and can be administered prior to, concurrently with or subsequent to antigen administration.

The invention also provides a method of generating large quantities of antigen-presenting dendritic cells ex vivo. Following collection of the patient's CD34<sup>+</sup> hematopoietic progenitors and stem cells, flt3-ligand can be used to expand such cells in vitro and to drive such CD34<sup>+</sup> cells to differentiate into dendritic cells. The resulting collection of dendritic cells is then exposed to an antigen and allowed to process and present the antigen in vitro (this procedure is sometimes referred to in the art as "antigen-pulsing"). An alternate method for preparing dendritic cells that present antigen is to transfect the dendritic cells with a gene encoding an antigen-specific polypeptide. Once the dendritic cells express the antigen, the antigen-presenting dendritic cells can be administered to a patient.

The invention also provides for the ex vivo preparation of antigen-specific T cells. Following the procedures described above for preparing large numbers of antigen-presenting dendritic cells ex vivo, the collected antigen-presenting dendritic cells are used to generate antigen-specific T cells from naive T cells that have been collected from a patient. After the antigen has been adequately presented to the T cells generated, the antigen-specific T cells can be administered to the patient.

The invention also provides a method of augmenting an immune response in a patient that has an infectious disease wherein the method comprises the step of administering an amount of flt3-ligand sufficient to increase the patient's number of dendritic cells.

The invention also provides a method of augmenting an immune response in a patient that has a cancerous or neoplastic disease wherein the method comprises the step of administering an amount of flt3-ligand sufficient to increase the patient's number of dendritic cells. Such method provides a means to enhance the patient's tumor-specific immune response.

A method for enhancing a patient's autoimmune tolerance wherein the method comprises the step of administering an amount of flt3-ligand sufficient to increase the patient's number of dendritic cells. Further included are methods for promoting survival of grafts and transplanted tissues and organs.

The methods of the invention can further comprise the use of an effective amount of a cytokine in sequential or concurrent combination with flt3-ligand. Such cytokines include, but are not limited to, interleukins ("ILs") IL-3 and IL-4, a colony stimulating factor ("CSF") selected from the group consisting of granulocyte macrophage colony stimulating factor ("GM-CSF") or GM-CSF/IL-3 fusions, or other cytokines such as TNF- $\alpha$  or c-kit ligand.

The invention further includes a dendritic cell expansion media comprising cell growth media, autologous serum, and flt3-ligand alone or in combination with a cytokine from the group listed above.

## DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to the use of flt3-ligand to generate large numbers of intermediate cell types from CD34+ hematopoietic progenitor cells and stem cells. Such intermediate cell types include myeloid cells, monocytic cells, macrophages, B cells and dendritic cells. The large numbers of these intermediate cell types are not naturally found in vivo and can be generated by administering flt3-ligand. Such enhancement in overall cell number can augment the immune response to antigen in the host. Another embodiment of the invention is the isolation and use of such intermediate cell types as antigen-presenting cells or the use thereof as vaccine adjuvants. The invention, while particularly focused on the embodiment concerning dendritic cells, is also applicable to myeloid, monocytic and macrophage cell types.

As used herein, the term "flt3-ligand" refers to a genus of polypeptides that are described in United States Patent No. 5,554,512, EP 0627487 A2 and in WO 94/28391, all incorporated herein by reference. A human flt3-ligand cDNA was deposited with the American Type Culture Collection, Rockville, Maryland, USA (ATCC) on August 6, 1993 and assigned accession number ATCC 69382. The deposit was made under the terms of the Budapest Treaty. Flt3-ligand can be made according to the methods described in the documents cited above.

The term "IL-3" refers to a genus of interleukin-3 polypeptides as described in U.S. Patent No. 5,108,910, incorporated herein by reference. Such polypeptides include analogs that have amino acid sequences that are substantially similar to the native human interleukin-3 amino acid sequences disclosed, for example, in EP publ. Nos. 275,598 and 282,185, each incorporated herein by reference. The term "IL-3" also includes analogs and alleles of IL-3 molecules that exhibit at least some of the biological activity in common with native human IL-3. Exemplary analogs of IL-3 are disclosed in EP Publ. No. 282,185. Other forms of IL-3 include human IL-3[Pro<sup>8</sup>Asp<sup>15</sup>Asp<sup>70</sup>], human IL-3[Ser<sup>8</sup>Asp<sup>15</sup>Asp<sup>70</sup>] and human IL-3[Ser<sup>8</sup>]. A DNA sequence encoding human IL-3 protein suitable for use in the invention is publicly available from the American Type Culture Collection (ATCC) under accession number ATCC 67747. The nomenclature used herein with respect to amino acid sequences in brackets designates which amino acids differ from the native human form. For example, human IL-3[Ser<sup>8</sup>Asp<sup>15</sup>Asp<sup>70</sup>] refers to a human IL-3 protein in which amino acid 8 has been changed to a serine residue, amino acid 15 has been changed to an aspartic acid residue and the amino acid 70 has been changed to an aspartic acid residue.

The term "IL-4" refers to a polypeptide as described in Mosley et al., *Cell* 59:335 (1989), Idzerda et al., *J. Exp. Med.* 171:861 (1990) and Galizzi et al., *Intl. Immunol.* 2:669 (1990), each of which is incorporated herein by reference. Such IL-4 polypeptide includes analogs that have an amino acid sequence that is substantially similar to the native human IL-4 amino acid sequences described in Mosley et al., Idzerda et al., and Galizzi et al. and which are biologically active in that they are capable of binding to a IL-4 receptor, transducing a biological signal initiated by binding IL-4 receptor, or cross-reacting with anti-IL-4 antibodies. The term "IL-4" also includes analogs of native human IL-4 molecules sufficient to retain biological activity of native human IL-4.

As used herein, "GM-CSF" refers to a genus of proteins as described in U.S. Patent Nos. 5,108,910, and 5,229,496 each of which is incorporated herein by reference. Such proteins include analogs that have an amino acid sequence that is substantially similar to native human GM-CSF amino acid sequences (e.g., as publicly available ATCC 53157 or ATCC 39900), and which are biologically active in that they are capable of binding to a GM-CSF receptor, transducing a biological signal initiated by binding GM-CSF receptor, or cross-reacting with anti-GM-CSF antibodies. Amino acid sequences are disclosed, for example in Anderson, et al., *Proc. Natl. Acad. Sci., USA* 82:6250 (1985). Commercially available GM-CSF (sargramostim, Leukine<sup>®</sup>) is obtainable from Immunex Corp., Seattle, WA). The term "GM-CSF" also includes analogs of the native human GM-CSF molecules described in U.S. Patent Nos. 5,108,910, and 5,229,496 sufficient to retain biological activity of native human GM-CSF. Exemplary analogs of GM-CSF include, for example,

those described in EP Publ. No. 212914 and WO 89/03881, each of which is incorporated herein by reference. Other analogs of GM-CSF also may be used to construct fusion proteins with IL-3. A DNA sequence encoding a particularly preferred GM-CSF protein having potential glycosylation sites removed is publicly available from the ATCC under  
5 accession numbers ATCC 67231.

The term "GM-CSF/IL-3 fusion protein" means a C-terminal to N-terminal fusion of GM-CSF and IL-3. The fusion proteins are known and are described in U.S. Patent Nos. 5,199,942, 5,108,910 and 5,073,627, each of which is incorporated herein by reference. A preferred fusion protein is PIXY321 as described in US Patent No.  
10 5,199,942.

The term "c-kit ligand" also known as Mast Cell Growth Factor (MGF), Steel Factor or Stem Cell Factor (SCF), refers to a polypeptide described in EP 423,980, which is incorporated herein by reference, and that claims priority from U.S. Patent application Serial No. 589,701, filed October 1, 1990. Such c-kit ligand polypeptide includes analogs  
15 that have an amino acid sequence that is substantially similar to the native human c-kit ligand amino acid sequences described in EP 423,980 and which are biologically active in that they are capable of binding to a c-kit receptor, transducing a biological signal initiated by binding c-kit receptor, or cross-reacting with anti-c-kit ligand antibodies. The term "c-kit ligand" also includes analogs of native human c-kit ligand molecules sufficient to retain  
20 biological activity of native human c-kit ligand.

The term "adjuvant" refers to a substance that enhances, augments or potentiates the host's immune response to a vaccine antigen.

The procedure for "ex vivo expansion" of hematopoietic stem and progenitor cells is described in U.S. Patent No. 5,199,942, incorporated herein by reference. Briefly, the  
25 term means a method comprising: (1) collecting CD34<sup>+</sup> hematopoietic stem and progenitor cells from a patient from peripheral blood harvest or bone marrow explants; and (2) expanding such cells *ex vivo*. In addition to the cellular growth factors described in Patent 5,199,942, other factors such as flt3-ligand, IL-1, IL-3, c-kit ligand, can be used.

The term "immunogenicity" means relative effectiveness of an immunogen or  
30 antigen to induce an immune response.

The term "substantially similar" means a variant amino acid sequence preferably that is at least 80% identical to a native amino acid sequence, most preferably at least 90% identical. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al.  
35 (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman



(*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds.,  
5 *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Variants may comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative  
10 substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Naturally occurring variants are also encompassed by the invention.  
15 Examples of such variants are proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the native protein, wherein the native biological property is retained.

As used herein, "vaccine" means an organism or material that contains an antigen in an innocuous form. The vaccine is designed to trigger an immunoprotective response. The  
20 vaccine may be recombinant or non-recombinant. When inoculated into a non-immune host, the vaccine will provoke active immunity to the organism or material, but will not cause disease. Vaccines may take the form, for example, of a toxoid, which is defined as a toxin that has been detoxified but that still retains its major immunogenic determinants; or a killed organism, such as typhoid, cholera and poliomyelitis; or attenuated organisms, that  
25 are the live, but non-virulent, forms of pathogens, or it may be antigen encoded by such organism, or it may be a live tumor cell or an antigen present on a tumor cell.

A variety of cell selection techniques are known for identifying and separating CD34<sup>+</sup> hematopoietic stem or progenitor cells from a population of cells. Methods and materials for identifying and selecting such cell types are known. For example,  
30 monoclonal antibodies can be used to bind to a marker protein or surface antigen protein found on stem or progenitor cells. Such markers or cell surface antigens for hematopoietic stem cells include CD34 and Thy-1. In one method, antibodies are fixed to a surface, for example, glass beads, and contacted with a mixture of cells suspected of containing stem cells. This permits the antibodies to bind and secure the stem cells to the glass beads.  
35 Alternatively, the antibodies can be incubated with the cell mixture and the resulting combination contacted with a surface having an affinity for the antibody-cell complex. Undesired cells and cell matter are removed providing a relatively pure population of stem

cells. Stem or progenitor cells having the CD34 marker constitute only about 1% to 3% of the mononuclear cells in the bone marrow. The amount of CD34<sup>+</sup> stem or progenitor cells in the peripheral blood is approximately 10- to 100-fold less than in bone marrow.

5 With regard to the particular aspects of the invention, choosing suitable stem or progenitor cell selection means will depend on the desired phenotype of the cell to be isolated. Hematopoietic stem cells are selectable by virtue of their physical characteristics, such as expressing the membrane-bound flt3 receptor, or having the following cellular markers: CD34 or Thy-1. Monoclonal antibodies that recognize any of these antigens have been described in U.S. Patent No. 4,714,680 (anti-My-10) incorporated herein by  
10 reference, anti-CD34 is commercially available from Becton Dickinson, Franklin Lakes, NJ), and anti-Thy-1 monoclonal antibodies can be readily generated using the methods described by Dalchau et al., *J. Exp. Med.* 149:576 (1979), incorporated herein by reference. A flt3 receptor binding protein also may be used, such as anti-flt3 monoclonal antibodies or the flt3-ligand. The cell binding protein is brought into contact with the  
15 collected cell mixture and the combination is allowed to incubate for a period of time sufficient to permit the binding of the desired cell to the cell binding protein.

An alternative means of selecting the quiescent stem cells is to induce cell death in the dividing, more lineage-committed, cell types using an antimetabolite such as 5-fluorouracil (5-FU) or an alkylating agent such as 4-hydroxycyclophosphamide (4-HC).  
20 The non-quiescent cells are stimulated to proliferate and differentiate by the addition of growth factors that have little or no effect on the stem cells, causing the non-stem cells to proliferate and differentiate and making them more vulnerable to the cytotoxic effects of 5-FU or 4-HC. See Berardi et al., *Science*, 267:104 (1995), which is incorporated herein by reference.

25 Isolation of the hematopoietic stem or progenitor cells can be performed by using, for example, affinity chromatography, antibody-coated magnetic beads, or antibodies fixed to a solid matrix, such as glass beads, flasks, etc. Antibodies that recognize a stem or progenitor cell surface marker can be fused or conjugated to other chemical moieties such as biotin - which can be removed with an avidin or a streptavidin moiety secured to a solid  
30 support; fluorochromes useful in fluorescence activated cell sorting (FACS), or the like. Preferably, isolation is accomplished by an immunoaffinity column. Immunoaffinity columns can take any form, but usually comprise a packed bed reactor. The packed bed in these bioreactors is preferably made of a porous material having a substantially uniform coating of a substrate. The porous material, which provides a high surface area-to-volume  
35 ratio, allows for the cell mixture to flow over a large contact area while not impeding the flow of cells out of the bed. Typical substrates include avidin and streptavidin, while other conventional substrates can be used. The substrate should, either by its own properties, or

by the addition of a chemical moiety, display high-affinity for a moiety found on the cell-binding protein such as a monoclonal antibody. The monoclonal antibodies recognize a cell surface antigen on the cells to be separated, and are typically further modified to present a biotin moiety. It is well-known that biotin has a high affinity for avidin, and the affinity of these substances thereby removably secures the monoclonal antibody to the surface of the packed bed. Such columns are well known in the art, see Berenson, et al., *J. Cell Biochem.*, 10D:239 (1986). The column is washed with a PBS solution to remove unbound material. Target cells can be released from the beads using conventional methods. Immunoaffinity columns of the type described above that utilize biotinylated anti-CD34 monoclonal antibodies secured to an avidin-coated packed bed are described for example, in PCT Publ. No. WO 93/08268. A variation of this method utilizes cell binding proteins, such as the monoclonal antibodies or flt3-ligand as described above, removably-secured to a fixed surface in the isolating means. The bound cell binding protein then is contacted with the collected cell mixture and allowed to incubate for a period of time sufficient to permit isolation of the desired cells.

Alternatively, the monoclonal antibodies that recognize the cell surface antigens can be labeled with a fluorescent label, e.g., chromophore or fluorophore, and separated by cell sorting according to the presence of absence or the amount of labeled product.

The collected CD34<sup>+</sup> cells are then exposed to either flt3-ligand alone or flt3-ligand in concurrent or sequential combination with one or more of the following cytokines: GM-CSF, TNF- $\alpha$ , IL-3, IL-4, c-kit-ligand or GM-CSF/IL-3 fusion proteins. CD34<sup>+</sup> cells then are allowed to differentiate and commit to cells of the dendritic lineage. The dendritic cells are collected and can either be (a) administered to a patient in order to augment the immune system and T-cell mediated or B-cell mediated immune responses to antigen, (b) exposed to an antigen prior to administration of the dendritic cells into a patient, (c) transfected with a gene encoding an antigen-specific polypeptide or (d) exposed to an antigen and then allowed to process and present the antigen, ex vivo, to T-cells collected from the patient followed by administration of the antigen-specific T-cells to the patient.

More specifically, the invention provides for the use of an effective amount of flt3-ligand to increase or mobilize dendritic cells in vivo, for example, in the patient's peripheral blood or other tissue or organs, such as the spleen. By increasing the quantity of the patient's dendritic cells, such cells may themselves be used to present antigen to T cells. For example, the antigen may be one that already exists within the patient, such as a tumor antigen, or a bacterial or viral antigen. Flt3-ligand may be used, therefore, to boost the patient's lymphocyte-mediated (e.g., T cell and B cell mediated) or myeloid-mediated immune response to the already present antigens thus potentially enabling a more effective antigen-presentation to the patient's T cells. Alternatively, flt3-ligand may be administered

prior to, concurrently with or subsequent to administration of an antigen to a patient for immunization purposes. Thus, as a vaccine adjuvant, flt3-ligand can generate large quantities of dendritic cells in vivo to more effectively present the antigen. The overall response is a stronger and improved immune response and more effective immunization to the antigen.

The systemic administration of flt3-ligand not only is effective as a vaccine adjuvant, but as discussed supra., is effective in augmenting an immune response against previously existing antigens. For example, the inventors have shown that flt3-ligand administration to tumor-bearing mice results in at least a significant decrease in the growth rate of the tumor, and can result in tumor rejection in a large proportion of the mice. The data are presented in more detail in Example 3. Flt3-ligand therefore is an important cytokine in the generation of an effective immune response in vivo against antigen.

Because of its ability to generate dendritic cells, flt3-ligand also finds use in promoting the survival of transplanted tissue or organs. When allogeneic organs or other tissue is transplanted into a host they can transfer stem cells, immature dendritic cells, and mature dendritic cells from the donor. These cells are called passenger cells and such cells can graft into the hematopoietic system of the host. Additionally, stem cells, immature dendritic cells, and mature dendritic cells from the host may graft to the donor organ or tissue. It is possible then to establish a tolerance between the graft and the host since the immature dendritic cells from the host and donor tissue interact with T-cells from the "other side." Such interaction may include the deletion of T-cells that recognize the major histocompatibility complex (MHC) that the dendritic cells express. In this way, the donor cells are "screened" so that they fail to recognize and react against the host (i.e., no graft versus host disease) and the host T-cells are screened so that they fail to recognize and react against the graft (i.e., no graft rejection). Thus, a mutual tolerance can be achieved, and the graft acceptance is improved. Administration of flt3-ligand to the host or donor prior to transplantation would generate increased numbers of dendritic cells in such host or donor and permit increased tolerance and survival of the graft.

For the growth and culture of dendritic cells, a variety of growth and culture media can be used, and the composition of such media can be readily determined by a person having ordinary skill in the art. Suitable growth media are solutions containing nutrients or metabolic additives, and include those that are serum-depleted or serum-based. Representative examples of growth media are RPMI, TC 199, Iscoves modified Dulbecco's medium (Iscove, et al., *F.J. Exp. Med.*, 147:923 (1978)), DMEM, Fischer's, alpha medium, NCTC, F-10, Leibovitz's L-15, MEM and McCoy's. Particular examples of nutrients that will be readily apparent to the skilled artisan include, serum albumin, transferrin, lipids, cholesterol, a reducing agent such as 2-mercaptoethanol or

monothioglycerol, pyruvate, butyrate, and a glucocorticoid such as hydrocortisone 2-hemisuccinate. More particularly, the standard media includes an energy source, vitamins or other cell-supporting organic compounds, a buffer such as HEPES, Tris, that act to stabilize the pH of the media, various inorganic salts. Particular reference is made to PCT  
5 Publ. No. WO 95/00632, wherein a variety of serum-free cellular growth media is described, such disclosure is incorporated herein by reference.

For any of the ex vivo methods of the invention, peripheral blood progenitor cells (PBPC) and peripheral blood stem cells (PBSC) are collected using apheresis procedures known in the art. See, for example, Bishop et al., *Blood*, vol. 83, No. 2, pp. 610-616  
10 (1994). Briefly, PBPC and PBSC are collected using conventional devices, for example, a Haemonetics Model V50 apheresis device (Haemonetics, Braintree, MA). Four-hour collections are performed typically no more than five times weekly until, for example, approximately  $6.5 \times 10^8$  mononuclear cells (MNC)/kg patient are collected. The cells are suspended in standard media and then centrifuged to remove red blood cells and  
15 neutrophils. Cells located at the interface between the two phases (also known in the art as the buffy coat) are withdrawn and resuspended in HBSS. The suspended cells are predominantly mononuclear and a substantial portion of the cell mixture are early stem cells. The resulting stem cell suspension then can be contacted with biotinylated anti-CD34 monoclonal antibodies or other cell-binding means. The contacting period is maintained for  
20 a sufficient time to allow substantial interaction between the anti-CD34 monoclonal antibodies and the CD34 antigens on the stem cell surface. Typically, times of at least one hour are sufficient. The cell suspension then is brought into contact with the isolating means provided in the kit. The isolating means can comprise a column packed with avidin-coated beads. Such columns are well known in the art, see Berenson, et al., *J. Cell*  
25 *Biochem.*, 10D:239 (1986). The column is washed with a PBS solution to remove unbound material. Target stem cells can be released from the beads and from anti-CD34 monoclonal antibody using conventional methods. The stem cells obtained in this manner can be frozen in a controlled rate freezer (e.g., Cryo-Med, Mt. Clemens, MI), then stored in the vapor phase of liquid nitrogen. Ten percent dimethylsulfoxide can be used as a  
30 cryoprotectant. After all collections from the donor have been made, the stem cells are thawed and pooled. Aliquots containing stem cells, growth medium, such as McCoy's 5A medium, 0.3% agar, and at least one of the expansion factors: recombinant human GM-CSF, IL-3, recombinant human flt3-ligand, and recombinant human GM-CSF/IL-3 fusion molecules (PIXY321) at concentrations of approximately 200 U/mL, are cultured and  
35 expanded at 37 °C in 5% CO<sub>2</sub> in fully humidified air for 14 days. Optionally, human IL-1 $\alpha$  or IL-4 may be added to the cultures. The most preferred combination of expansion factors comprises flt3-ligand plus either IL-3 or a GM-CSF/IL-3 fusion protein.

For *in vivo* administration to humans, flt3-ligand can be formulated according to known methods used to prepare pharmaceutically useful compositions. Flt3-liigand can be combined in admixture, either as the sole active material or with other known active materials, with pharmaceutically suitable diluents (e.g., Tris-HCl, acetate, phosphate),  
5 preservatives (e.g., Thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed. 1980, Mack Publishing Co. In addition, such compositions can contain flt3-ligand complexed with polyethylene glycol (PEG),  
10 metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of flt3-ligand.

Flt3-ligand can be administered topically, parenterally, or by inhalation. The term  
15 "parenteral" includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. These compositions will typically contain an effective amount of the flt3-ligand, alone or in combination with an effective amount of any other active material. Such dosages and desired drug concentrations contained in the compositions may vary depending upon many factors, including the intended use, patient's  
20 body weight and age, and route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration can be performed according to art-accepted practices. Keeping the above description in mind, typical dosages of flt3-ligand may range from about 10 µg per square meter to about 1000 µg per square meter. A preferred dose range is on the order of about 100 µg per square  
25 meter to about 300 µg per square meter.

In addition to the above, the following examples are provided to illustrate particular embodiments and not to limit the scope of the invention.

### 30 EXAMPLE 1 Generation of Dendritic Cells

This Example describes a method for using flt3-ligand to generate large numbers of dendritic cells *ex vivo*. Cells having the CD34<sup>+</sup> phenotype are isolated as described above, for example, first by generating a buffy coat of cells using a procedure described supra.  
35 Cells from the buffy coat are then incubated with a CD34 specific monoclonal antibody. The CD34<sup>+</sup> cells which are selected then are cultured in McCoy's enhanced media with 20 ng/ml each of GM-CSF, IL-4, TNF-α, or 100 ng/ml flt3-ligand or c-kit ligand. The culture is continued for approximately two weeks at 37 °C in 10% CO<sub>2</sub> in humid air. Cells

then are sorted by flow cytometry for CD1a<sup>+</sup> and HLA-DR<sup>+</sup> expression. The combination of GM-CSF, IL-4 and TNF- $\alpha$ , resulted in a six to seven-fold increase in the number of cells obtained after two weeks of culture. The combination of flt3-ligand and c-kit ligand resulted in an additive 12-13-fold increase in absolute cell numbers. This correlated with an 18-fold expansion with either flt3-ligand or c-kit ligand or to a 34-fold expansion with the combination of flt3-ligand and c-kit ligand. Phenotypic analysis of the cells showed that between 60-70% of the cells were HLA-DR<sup>+</sup>, CD86<sup>+</sup>, with 40-50% of the cells expressing CD1a in all factor combinations examined. The addition of flt3-ligand increased the absolute number of CD1a<sup>+</sup> cells by 5-fold. c-Kit ligand increased those cells by 6.7-fold and the combination of flt3-ligand and c-kit ligand by 11-fold. Functional analysis of the resultant cells in an MLR revealed that the presence of flt3-ligand or c-kit ligand did not affect the stimulatory capacity of the resultant dendritic cells while increasing the numbers attained.

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## **EXAMPLE 2** **Use of Flt3-L in Dendritic Cell Expansion**

This Example describes a method for using flt3-ligand for dendritic cell expansion. Prior to cell collection, it may be desirable to mobilize or increase the numbers of circulating PBPC and PBSC. Mobilization can improve PBPC and PBSC collection, and is achievable through the intravenous administration of flt3-ligand or sargramostim (Leukine<sup>®</sup>, Immunex Corporation, Seattle, Washington) to the patients prior to collection of such cells. Other growth factors such as CSF-1, GM-CSF, c-kit ligand, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF, FGF and combinations thereof, can be likewise administered in sequence, or in concurrent combination with flt3-ligand. Mobilized or non-mobilized PBPC and PBSC are collected using apheresis procedures known in the art. See, for example, Bishop et al., *Blood*, vol. 83, No. 2, pp. 610-616 (1994). Briefly, PBPC and PBSC are collected using conventional devices, for example, a Haemonetics Model V50 apheresis device (Haemonetics, Braintree, MA). Four-hour collections are performed typically no more than five times weekly until approximately  $6.5 \times 10^8$  mononuclear cells (MNC)/kg patient are collected. Aliquots of collected PBPC and PBSC are assayed for granulocyte-macrophage colony-forming unit (CFU-GM) content by diluting approximately 1:6 with Hank's balanced salt solution without calcium or magnesium (HBSS) and layering over lymphocyte separation medium (Organon Teknika, Durham, North Carolina). Following centrifugation, MNC at the interface are collected, washed and resuspended in HBSS. One milliliter aliquots containing approximately

300,000 MNC, modified McCoy's 5A medium, 0.3% agar, 200 U/mL recombinant human GM-CSF, 200 u/mL recombinant human IL-3, and 200 u/mL recombinant human G-CSF are cultured at 37 °C in 5% CO<sub>2</sub> in fully humidified air for 14 days. Optionally, flt3-ligand or GM-CSF/IL-3 fusion molecules (PIXY 321) may be added to the cultures. These cultures are stained with Wright's stain, and CFU-GM colonies are scored using a dissecting microscope (Ward et al., *Exp. Hematol.*, 16:358 (1988). Alternatively, CFU-GM colonies can be assayed using the CD34/CD33 flow cytometry method of Siena et al., *Blood*, Vol. 77, No. 2, pp 400-409 (1991), or any other method known in the art.

CFU-GM containing cultures are frozen in a controlled rate freezer (e.g., Cryo-Med, Mt. Clemens, MI), then stored in the vapor phase of liquid nitrogen. Ten percent dimethylsulfoxide can be used as a cryoprotectant. After all collections from the patient have been made, CFU-GM containing cultures are thawed and pooled. The thawed cell collection is contacted with flt3-ligand either alone, sequentially or in concurrent combination with other cytokines listed above. Such exposure to flt3-ligand will drive the CFU-GM to dendritic cell lineage. The dendritic cells are reinfused intravenously to the patient.

### **EXAMPLE 3**

#### **Use of Flt3-L in Augmenting Anti-tumor Immune Responses**

This Example describes a method for using flt3-L to augment anti-tumor immune responses in vivo. Female C57BL/10J (B10) mice (The Jackson Laboratory, Bar Harbor, ME) were injected with  $5 \times 10^5$  viable B10.2 or B10.5 fibrosarcoma tumor cells by intradermal injection in a midline ventral position in a total volume of 50 $\mu$ l. The fibrosarcoma B10.2 and B10.5 lines are of B10 origin and have been described previously, see Lynch et al., *Euro. J. Immunol.*, 21:1403 (1991) incorporated herein by reference. The fibrosarcoma B10.2 line was induced by subcutaneous implantation of a paraffin pellet containing 5 mg of methylcholanthrene, and the B10.5 line was induced by chronic exposure to ultraviolet radiation. The tumor cell lines were maintained in vitro in  $\alpha$ -modified MEM containing 5% FBS, 2mM L-glutamine, 50U/ml penicillin and 50  $\mu$ g/ml streptomycin. Recombinant human flt3-L (10 $\mu$ g/injection) was administered on a daily basis over a 19-day period (unless otherwise noted) by subcutaneous injection in a total volume of 100  $\mu$ l. Control mice were similarly injected with a similar volume of buffer containing 100 ng MSA. Tumor growth rates were determined by plotting the tumor size versus time after tumor challenge. Tumor size was calculated as the product of two perpendicular diameters, measured by calipers, and is expressed as the mean tumor size of only those mice bearing a tumor within a particular treatment group. The number of mice



bearing tumors compared to the number challenged for each treatment group at the termination of an experiment are shown in the data below.

From Table I, the data is a compilation of six different experiments wherein tumor-bearing mice were either treated with flt3-ligand or MSA. Complete tumor regression was observed in 19 of 50 flt3-ligand treated mice compared to 1 of 30 in MSA-treated mice ( $p < 0.0001$  using Fishers Exact Test). The observation that the rate of tumor growth in flt3-ligand treated mice (mean tumor size in tumor-bearing mice at week 5 post-tumor challenge was  $60 \pm 8 \text{ mm}^2$ ) was significantly reduced compared to MSA-treated mice (mean tumor size at week 5 post-tumor challenge was  $185 \pm 17 \text{ mm}^2$ ) was also confirmed ( $p.0001$  by Analysis of Variance).

**TABLE I**  
Fibrosarcoma +/- Flt3-L Composite of Six Experiments  
Tumor Size ( $\text{mm}^2$ )

Weeks Post Tumor Challenge	MSA Control (100ng/day)	Standard Error	Flt3-L (10 $\mu$ g/day)	Standard Error
0	0	0	0	0
1	25	2.6	24	2.2
2	62	7.5	49	3.6
3	98	10.6	49	3.9
4	149	14.5	50	5
5	185	16.8	60	8.4

Tumor size was sharply retarded with flt3-ligand compared to the control. Therefore, the data show that flt3-ligand is an important cytokine in the augmentation of the immune response against foreign antigens, and in particular against cancer.

What is claimed is:

1. A method for augmenting an immune response in a patient comprising the step of administering an amount of flt3-ligand to the patient sufficient to generate an increase in the number of the patient's dendritic cells.
2. A method according to claim 1, further comprising the step of administering one or more of the molecules selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.
3. A method for augmenting an immune response in a patient having an infectious disease, comprising the step of administering of administering flt3-ligand in an amount sufficient to generate an increase in the number of the patient's dendritic cells.
4. A method according to claim 3, further comprising the step of administering one or more of the molecules selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.
5. A method according to claim 3, wherein the infectious disease is HIV.
6. A method for augmenting an immune response in a patient having a cancerous or neoplastic disease, comprising the step of administering flt3-ligand in an amount sufficient to generate an increase in the number of the patient's dendritic cells.
7. A method according to claim 6, further comprising the step of administering one or more of the molecules selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.
8. A preparation of dendritic cells having at least two cell surface markers selected from the group consisting of CD1a, HLA-DR and CD86, produced by contacting hematopoietic stem or progenitor cells with flt3-ligand.
9. A dendritic cell preparation according to claim 8 produced further by contacting the hematopoietic stem or progenitor cells with a molecule selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.

10. An antigen-expressing dendritic cell population produced by the process of
  - (a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;
  - (b) either (i) exposing the dendritic cells to an antigen-specific peptide or (ii) transfecting the dendritic cells with a gene encoding an antigen-specific peptide;
  - (c) allowing the dendritic cells to process and express the antigen; and
  - (d) purifying the antigen-expressing dendritic cells.
11. A dendritic cell population according to claim 10 wherein step (a) of the process further comprises contacting the hematopoietic stem or progenitor cells with a molecule selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.
12. A method of driving hematopoietic stem or progenitor cells to a dendritic cell lineage comprising contacting such hematopoietic stem or progenitor cells with flt3-ligand.
13. A method of preparing an antigen-presenting dendritic cell population comprising the steps of:
  - (a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;
  - (b) either (i) exposing the dendritic cells to an antigen-specific peptide or (ii) transfecting the dendritic cells with a gene encoding an antigen-specific peptide;
  - (c) allowing the dendritic cells to process and express the antigen; and
  - (d) purifying the antigen-expressing dendritic cells.
14. A method according to claim 13, wherein step (a) further comprises contacting the hematopoietic stem or progenitor cells with a molecule selected from the group consisting of GM-CSF, IL-4, TNF- $\alpha$ , IL-3, c-kit ligand, and fusions of GM-CSF and IL-3.
15. A method of preparing antigen-specific T cells comprising the steps of:
  - (a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;

- (b) either (i) exposing the dendritic cells to an antigen-specific peptide or (ii) transfecting the dendritic cells with a gene encoding an antigen-specific peptide;
- (c) allowing the dendritic cells to process and express the antigen; and
- (d) allowing the dendritic cells to present the antigen to T cells.

16. A method of enhancing a mammal's immune response to a vaccine antigen, comprising the steps of administering to such mammal an immunogenic amount of the vaccine antigen and an immunogenicity-augmenting amount of flt3-ligand in concurrent or sequential combination with such vaccine antigen.

17. A vaccine adjuvant comprising a molecule selected from the group consisting of c-kit ligand and flt3-ligand.

18. A method for inducing tolerance of graft tissue in a host, comprising administering flt3-ligand to the host in an amount sufficient to increase the number of dendritic cells.

19. A dendritic cell expansion media comprising an effective amount of flt3-ligand and a cytokine selected from the group consisting of IL-3, IL-4, GM-CSF, TNF, C-Kit ligand and GM-CSF/IL-3 fusion proteins.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/15990

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 45/05, 39/12, 39/02, 39/00; A01N 1/02; C12N 5/00

US CL : 424/85.1, 204.1, 234.1, 277.1; 435/2, 240.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 204.1, 234.1, 277.1; 435/2, 240.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WPIDS, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUDAK et al. FLT3/FLK2 ligand promotes the growth of murine stem cells and the expansion of colony-forming cells and spleen colony-forming units. Blood. 15 May 1995, Vol. 85, No. 10, pages 2747-2755, see entire document.	1-19
Y	HANNUM et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature. 14 April 1994, Vol. 368, pages 643-648, see entire document.	1-19
Y	MUENCH et al. FLK-2/FLT-3 ligand regulates the growth of early myeloid progenitors isolated from human fetal liver. Blood. 15 February 1995, Vol. 85, pages 963-972, see entire document.	1-19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

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## INTERNATIONAL SEARCH REPORT

International application No.

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STEINMAN. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 1991, Vol. 9, pages 271-296, see entire document.	1-19
Y	MACATONIA et al. Primary proliferative and cytotoxic T-cell responses to HIV induced in vitro by human dendritic cells. Immunology. 1991, Vol. 74, pages 399-406, see entire document.	1-19
Y	PANCHOLI et al. Dendritic cells efficiently immunoselect mycobacterial-reactive T cells in human blood, including clonable antigen-reactive precursors. Immunology. 1992, Vol. 76, pages 217-224, see entire document.	1-19
Y	INABA et al. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J. Exp. Med. August 1990, Vol. 172, pages 631-640, see entire document.	1-19
Y	BUJDOSO et al. Afferent lymph dendritic cells: a model for antigen capture and presentation in vivo. Intern. Rev. Immunol. 1990, Vol. 6, pages 177-186, see entire document.	1-19
Y	JAFFE. Review of human dendritic cells: Isolation and culture from precursors. Pediatric Pathology. 1993, Vol. 13, pages 821-837, see entire document.	1-19
Y	BERNSTEIN et al. Isolation of human hematopoietic stem cells. Blood Cells. 1994, Vol. 20, pages 15-24, see entire document.	1-19
Y	YOUNG et al. Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. J. Exp. Med. October 1995, Vol. 182, pages 1111-1120, see entire document.	1-19
Y	INABA et al. Dendritic cell progenitors phagocytose particulates, including Bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J. Exp. Med. August 1993, Vol. 178, pages 479-488, see entire document.	1-19